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Timed artificial insemination should be performed early when used norgestomet ear implants are applied for synchronizing ovulation in beef heifers

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ABSTRACT

The present study evaluated the effect of the type of norgestomet ear implant (new vs. used) on the ovarian follicular response (experiment 1) and pregnancy per artificial insemination (AI) (P/AI; experiment 2) of beef heifers subjected to an estradiol plus progestin timed artificial insemination (TAI) program. In experiment 1, 57 cyclic beef heifers were randomly assigned to one of two groups according to the type (new or previously used for 9 days) of norgestomet ear (NORG) implant. At the time of NORG implant insertion, the heifers were treated with 2 mg of intramuscular estradiol benzoate. Eight days later, the NORG implants were removed, and the heifers received an intramuscular administration of 150 μg of D-cloprostenol, 300 IU of equine chorionic gonadotropin, and 0.5 mg of estradiol cypionate. The heifers had their ovaries scanned every 12 hours from the time of NORG implant removal to 96 hours after verifying the occurrence and timing of ovulation. No difference (P = 0.89) was observed in the ovulation rates between the two treatments (new = 80.0%; 24/30 vs. used = 81.5%; 22/27). However, the heifers treated with a used NORG implant had (P = 0.04) higher proportion (36.4%; 8/22) of early ovulation (between 36 and 48 hours after NORG implant removal) compared with the heifers treated with a new NORG implant (8.3%; 2/24). In experiment 2, at the beginning of the synchronization protocol, 416 beef heifers were randomly assigned into two groups, as described in the experiment 1. Two days after the NORG implant removal, the heifers were reassigned to be inseminated at 48 or 54 hours after NORG implant removal. There was an interaction (P = 0.03) between the type of NORG implant and the timing of TAI on P/AI. The timing of insemination only had an effect (P = 0.02) on the P/AI when the heifers were treated with a used NORG implant [(TAI 54 hours = 41.9% (44/105) vs. TAI 48 hours = 58.6% (58/99)]. In conclusion, beef heifers synchronized with a used NORG implant plus estradiol exhibited a higher proportion of earlier ovulations, and TAI in these heifers should be performed 48 hours after removal of used NORG implants.

1. Introduction

The genetic gain is an important goal in cow-calf operations to increase the production per area and animal productive capacity. This progress can be achieved using reproductive technologies, especially artificial insemination

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(AI). Thus, proportional enhancement of pregnant females using AI must be a target to reach a successful reproduction program by improving the service rates and pregnancy outcomes.

Despite commercial restrictions observed in some countries such as United States of America, New Zealand, and countries of European Union on the use of estradiol 17-beta and its ester-like derivatives to control the estrous and ovulation in cattle, its hormonal therapy can legally be used in South America. Furthermore, progesterone (P4) plus estradiol-based timed artificial insemination (TAI) protocol has been the most commercially used TAI synchronization protocol in Brazil [1–5]. In beef heifers, a common aspect among the estrus synchronization protocols for TAI is the insertion of an intravaginal device containing P4 or an ear implant containing norgestomet (NORG) plus administration of estradiol benzoate (EB; 2 mg intramuscular) on Day 0; an injection of prostaglandin (PG) $F_{2\alpha}$ on Day 8 or 9 at the moment of device withdrawal plus 300 to 400 IU of equine chorionic gonadotropin (eCG) [2,3,5,6]. Different ovulation inducers with similar efficiency can be used, such as estradiol cypionate (ECP; 0.5 mg intramuscular) at the moment of implant removal, EB (1.0 mg intramuscular) 24 hours after P4/progestin implant removal or GnRH at insemination [4]. TAI is generally performed 52 to 56 hours after device removal [1], and several experiments recently indicated the possibility of increasing this period to 48 to 60 hours after P4/progestin withdrawal [4,7,8].

Using a progestin/P4 implant more than once is a common practice to reduce the cost of estrus synchronization protocols for TAI. Previously used P4 implants provide a lower circulating concentration of P4 during the synchronization protocol [3,9,10]. The P4 has a significant effect on follicle development by regulating LH pulse frequency and indirectly controlling estradiol synthesis [11–14]. Lower circulating concentrations of P4 during the synchronization protocol could result in an increased frequency and amplitude of LH pulses, faster growth of the dominant follicle, and greater concentration of circulating estradiol during the proestrus [12,14,15]. The presence of a larger and mature dominant follicle leads to a short interval from the time of luteolysis or P4 source removal to the onset of estrus [12,16,17]. Therefore, heifers subjected to estrus synchronization protocols under lower levels of circulating P4/progestin (i.e., used implants) could be expected to have a larger follicle size at the time of implant removal, which could increase the occurrence of earlier ovulation, altering the window to perform TAI in cyclic beef heifers.

The optimal time at which insemination should take place relative to ovulation (Insemination-Oulation Interval) depends primarily on the lifespan of spermatozoa and on the viability of the oocyte in the female genital tract [18]. Several experiments have found that 6 hours is the minimum time needed for a viable sperm population capable of fertilization to pass through the oviduct [18–20]. Regarding the oocyte, the most desirable period for fertilization appears to be between 6 and 10 hours after ovulation [21], and the probability of conception decreases when AI is performed near the time of ovulation (less than 6–12 hours before ovulation; [22,23]). Additionally, the fertilization rate drastically decreases when AI occurs after ovulation [23].

Furthermore, the present study aimed to evaluate the timing of TAI (48 or 54 hours after NORG implant removal) according to the number of uses of the NORG implant (new or previously used for 9 days) on ovarian follicular dynamics (experiment 1) and pregnancy per AI (P/AI; experiment 2) in cyclic beef heifers. The hypothesis is that the optimum time to perform TAI depends on the NORG implant (new or used) used for the estrus synchronization protocol in beef heifers.

2. Materials and methods

2.1. Experiment 1: effect of a used NORG implant on ovarian follicular dynamics

2.1.1. Location and animals

This experiment was conducted in a state research farm (APTA—Alta Mogiana Regional Center) located in Colina, São Paulo, Brazil. All of the heifers were kept on *Brachiaria brizantha* pasture and given mineralized salt and free access to water. Data collection was performed during the 2010/2011 (group 1) and 2011/2012 (group 2) spring-summer (November–January) breeding seasons. A total of 57 cyclic Nelore (*Bos indicus*) beef heifers ranging between 20 and 24 months of age were assigned into two groups (group 1 [$n = 32$] and group 2 [$n = 25$]). The heifers were examined by transrectal ultrasonography to determine the presence of a CL on the first day of the synchronization protocol.

2.1.2. Experimental design

At a random stage of the estrous cycle, the heifers were randomly assigned to be treated with a new ($n = 30$) or a previously used NORG implant for 9 days ($n = 27$) (Crestar, MSD Animal Health, Boxmeer, Netherlands) and 2 mg of intramuscular EB (Estrogin, Farmavet, Sao Paulo, Brazil). After initial use, the used NORG implants were individually washed with water and soaked in a solution of chloride alkyl dimethyl benzyl ammonium (CB 30, Ouro Fino Agronegócio, São Paulo, Brazil) for ~10 minutes. Thereafter, the implants were dried using brown paper, thoroughly wrapped in aluminum paper, and stored at room temperature until use. Eight days after the insertion, the NORG implants were removed, and the heifers were given 150 μg of intramuscular D-cloprostenol (Preloban, MSD Animal Health), 300 IU of equine chorionic gonadotropin (eCG, Folligon, MSD Animal Health), and 0.5 mg of estradiol cypionate (ECP, Pfizer Animal Health, São Paulo, SP, Brazil).

2.2. Experiment 2: effect of a used NORG implant on pregnancy per AI

2.2.1. Location and animals

This experiment was conducted in a commercial beef farm located in Rio Verde do MT, Mato Grosso do Sul, Brazil. All of the heifers were kept on a *Brachiaria humidicula* pasture and given mineralized salt and free access to water. Data collection was performed during the 2010/2011 and 2011/2012 spring-summer (November–January) breeding seasons. A total of 416 cyclic Nelore (*B. indicus*) beef heifers aged between 20 and 30 months were used. The heifers were examined by transrectal ultrasonography to

determine the presence of a CL 45 days before and at the onset of the ovulation synchronization protocol. The body condition score was determined on the first day of the estrous synchronization protocol using a 1 to 5 scale (1 = emaciated, 5 = obese) [24].

2.2.2. Reproductive management and treatment

Forty-five days before the onset of the reproductive breeding season, all of the heifers were examined by ultrasound and classified for presence or absence of a CL (with a CL = 29.3%; 122/416 or without a CL = 70.7%; 294/416). Regardless of the presence of a CL, the heifers were stimulated with a previously used for 24 days P4 intravaginal device (CIDR, Pfizer Animal Health) that was kept for 10 days. At the time of P4 intravaginal device removal, the heifers received an injection of 0.5 mg of intramuscular ECP [25].

On the first day of the breeding season, heifers were examined by ultrasound and classified according to the presence (85.8%; 357/416) or absence (14.2%; 59/416) of a CL. Next, the heifers were divided according to the presence or absence of a CL and were randomly assigned to be treated with a new ($n = 212$) or a previously used for 9 days ($n = 204$) NORG implant and 2 mg of intramuscular EB. Eight days later (Day 8), the NORG implants were removed, and the heifers received 150 μg of intramuscular D-cloprostenol, 300 IU of eCG, and 0.5 mg of ECP. On the day of TAI, the females were newly reassigned to be inseminated at 48 ($n = 204$) or 54 hours ($n = 212$) after NORG implant removal (factorial 2 × 2). Thus, half of the heifers treated with a new NORG implant were timed for AI at 48 ($n = 105$) or 54 hours ($n = 107$), and half of the heifers treated with a previously used NORG implant received a TAI at 48 ($n = 99$) or 54 hours ($n = 105$) after NORG implant removal. Inseminations were performed by two technicians using frozen-thawed semen from two previously proven fertile bulls in the TAI program (the AI technicians and bulls used were equally distributed among the experimental groups).

2.3. Ultrasonography examination

In experiment 1, transrectal ultrasonography was performed using a 3.5/5.0/6.0/8.0-MHz linear-array transducer (8100, Chison Medical Imaging Co., China). Ovarian ultrasonographic examinations were performed twice daily from Days 8 to 12 to evaluate ovarian follicular dynamics and the interval from NORG implant removal to ovulation. In all of the ultrasound examinations, both ovaries were visualized to identify and measure the three largest follicles (LFs) present in each ovary. The diameter of the LF during NORG implant removal and 48 hours after were recorded. Ovulation was considered to have occurred when a large follicle, previously observed, was no longer present.

In experiment 2, a real-time ultrasonic scanner equipped with a 7.5-MHz linear transducer (CTS-3300V, SIUI, China) was used for the ultrasound examination 45 days before the breeding season, at onset of the synchronization protocol, and for a pregnancy diagnosis 30 days after TAI. The P/AI was defined as the number of pregnant heifers divided by the total number of heifers submitted to TAI in each group (new 48 hours, new 54 hours, used 48 hours, and used 54 hours). Detection of an embryonic vesicle with a viable embryo (presence of a heartbeat) was used as an indicator of pregnancy.

2.4. Statistical analysis

Statistical analysis was conducted using the PROC GLIMMIX of SAS System for Windows (SAS Institute Inc., Cary, NC, USA, 2003).

In experiment 1, the explanatory variables included in the statistical model were the type of NORG implant (new vs. used), group, and interactions. The dependent variables, such as LF at NORG implant removal, LF 48 hours after implant removal, and timing of ovulation relative to NORG implant removal, were tested according to their homogeneity and normality of variances using the Guide Data Analysis from SAS and subjected to transformation if necessary. Because Levene's test reveals the heterogeneity of variances, the median test was used to compare the intervals from NORG implant removal to ovulation. A binomial distribution was assumed for the categorical response variables. The ovulation rate and occurrence of earlier ovulation (ovulation occurring between 24 and 48 hours after NORG implant removal) were also analyzed, with the type of NORG implant, group, and interactions being included in the model.

In experiment 2, the variables initially included in the models were the presence of a CL 45 days before the breeding season, BCS, age on the first day of the ovulation synchronization protocol, type of NORG implant (new or used), and timing of TAI (48 or 54 hours after NORG implant removal). For the final logistic regression model, variables were removed through backward elimination, on the basis of the Wald's statistics criterion when $P > 0.20$. Variables included in the final model for analysis of the P/AI were the presence of a CL 45 days before breeding season, BCS at the first day of the TAI protocol, type of NORG implant used, timing of TAI, and interaction between the type of NORG implant and timing of TAI.

LSMeans ± SE are used to describe all of the response variables. $P \leq 0.05$ was considered to be statistically significant for the variables evaluated.

3. Results

3.1. Experiment 1: effect of a used NORG implant on ovarian follicular dynamics

There was no interaction between treatment and any of the response variables ($P > 0.05$). Additionally, no effect was found for BCS on the first day of synchronization and for the type of NORG implant on LF diameter at implant removal. Moreover, there were no significant effects on LF diameter at 24 or 48 hours after NORG implant removal and the timing and rate of ovulation (Table 1). However, there was an effect for the type of NORG implant on the occurrence of early ovulation ($P = 0.04$; Table 1).

3.2. Experiment 2: effect of a used NORG implant on pregnancy per AI

No effect of the BCS ($P = 0.12$) or age ($P = 0.39$) on the first day of synchronization was found for the P/AI. The

Table 1
Ovarian follicular dynamics of beef heifers synchronized with a progestin (new or previous used for 9 days) plus estradiol-based TAI synchronization protocol (experiment 1).

Item	Treatment[a]		P value
	New	Used	
Number of heifers	30	27	——
Body condition score at first day of synchronization treatment	2.9 ± 0.0	2.9 ± 0.0	0.22
LF diameter at NORG implant removal (mm)	8.3 ± 0.4	8.6 ± 0.4	0.62
LF diameter 24 hours after NORG implant removal (mm)	10.2 ± 0.4	10.4 ± 0.4	0.73
LF diameter 48 hours after NORG implant removal (mm)	11.3 ± 0.4	11.4 ± 0.5	0.87
Ovulation rate (%)	80.0	81.5	0.89
Timing of ovulation (h)[b]	67.7 ± 2.4	62.7 ± 2.5	0.16
Occurrence of earlier ovulation (%)[c]	8.3	36.4	0.04

Abbreviation: LF, largest ovarian follicle.
[a] Heifers at a random stage of the estrous cycle were treated with a new or previously used for 9 days norgestomet (NORG) ear implant and 2 mg of intramuscular estradiol benzoate. Eight days later, the NORG implants were removed, and all heifers received 150 μg of intramuscular D-cloprostenol, 300 IU of eCG, and 0.5 mg of ECP.
[b] Timing of ovulation relative to the NORG implant removal.
[c] Ovulation occurring from 24 to 48 hours after NORG implant removal.

overall mean age (months) and BCS were 25.7 ± 3.7 and 3.6 ± 0.2, respectively. There was also no effect based on the type of NORG implant (P = 0.48) and timing of TAI (P = 0.28). However, a significant effect of interaction (P = 0.03) between the type of NORG and timing of TAI on the P/AI was found (Fig. 1). In addition, heifers that presented with a CL 45 days before the onset of the breeding season had a greater (P = 0.03) P/AI (58.2%; 71/122) than heifers that did not have a CL at that time (45.6%; 134/294).

4. Discussion

The current study found that the most appropriate moment to perform TAI in beef heifers depends on the progestin implant (new or used) used for estrus synchronization, confirming the present hypothesis. Using a new NORG implant allows for insemination to be performed as traditionally recommended at 54 hours or earlier at 48 hours after NORG implant removal. In contrast, working with a used NORG implant, a lower P/AI is achieved when TAI is performed at 54 hours compared with 48 hours.

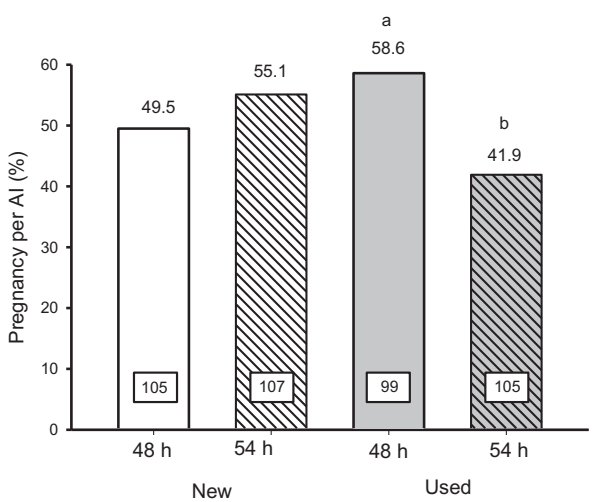

Fig. 1. Pregnancy per AI in beef heifers according to the type of norgestomet (NORG) ear implant (new or previously used for 9 days) and the timing of TAI (48 or 54 hours) relative to the NORG implant removal (experiment 2). There was an interaction between the type of NORG implant and the timing of TAI (P = 0.03). [a,b] The bars with different letters are different (P = 0.02).

AI should occur near the time of ovulation to maximize sperm access to the ovum but not so late that an aging ovum awaits sperm arrival [26]. The optimal AI time was between 12 and 24 hours before ovulation for the most desirable rate of fertilization, and 12 to 16 hours for the greatest percentage of high-quality embryos (89% of recovered embryos; [23]). More precisely, previous study found that an optimal pregnancy rate could be achieved when AI is performed 16.2 hours before ovulation [27]. In suckled beef *B. indicus* cows, an IOI of 5.4 hours had reduced P/AI when compared with an IOI of 15.3 hours [28]. Similarly, the P/AI achieved in the current study was lower in heifers treated with a used implant and inseminated at 54 hours. Furthermore, assuming similar moment of ovulation according the type of NORG implant observed in the experiment 1, might be possible that heifers inseminated at 54 hours in the experiment 2 had lower IOI than did heifers inseminated at 48 hours or those treated with a new NORG implant. Furthermore, a significant number of heifers treated with a used NORG implant could be already ovulated at 48 hours, and more than 6 hours after ovulation when they were inseminated 54 hours after NORG implant removal. Therefore, a lower P/AI in heifers treated with a used NORG implant and TAI at 54 hours could be explained by a reduced fertilization rate due to receiving TAI close to or after ovulation [22,23,29].

In the present study, the most appropriate time to perform the TAI in beef heifers was dependent on the type of NORG implant used during the synchronization protocol. In heifers subjected to the TAI protocol using a used NORG implant, a lower P/AI was observed when TAI was performed at 54 hours compared with 48 hours. In agreement with these findings, in suckled beef cows, Castro Júnior et al. [30], working with used NORG implants and ECP to induce ovulation, also found lower P/AI when inseminating 54 hours (38.5%) rather than 48 hours (49.5%) after implant removal. In addition, a higher pregnancy rate was detected in *Bos taurus* beef females treated with a once-used P4

device compared with a twice-used device (62.4% vs. 48.4%, respectively) when females were inseminated 52 to 56 hours after P4 removal [31].

Progesterone has a significant effect on follicle development by regulating LH pulse frequency and indirectly controlling estradiol synthesis [11–14]. Lower circulating P4 concentrations during the synchronization protocol could result in an increased frequency and amplitude of LH pulses, altered size or maturity of dominant follicle, and a greater concentration of circulating estradiol at the end of the treatment [12,14,15,17]. The presence of a larger, more mature dominant follicle at implant removal could lead to a short interval from P4/progestin source removal to the onset of estrus and ovulation [12,32]. Furthermore, in this study, in heifers treated with a used NORG implant, 36% of the animals that had already ovulated at 48 hours after implant removal, the fertilization rate drastically decreased when AI occurs after ovulation. This result is consistent with the findings of Roelofs et al. [23].

The data reported here indicated that heifers that are cyclic at least 45 days before the onset of a TAI synchronization protocol had a greater P/AI than heifers that became cyclic during the last 45 days before the onset of a TAI protocol. Previous reports have indicated that the pregnancy outcomes were greater in the third estrous cycle, rather than in the first, after the heifers achieved puberty [33]. These cited authors also reported a higher pregnancy rate in heifers bred during the third estrus than the pubertal estrus. In *B. indicus* heifers, previous studies have found that the presence of CL on the first day of the estradiol plus progestin-based TAI protocol is important to optimize fertility following AI [5]. Therefore, the present results suggest an important relationship between the previous cyclic status and the pregnancy outcomes of *B. indicus* beef heifers submitted to a progestin-based TAI protocol at the onset of breeding season.

4.1. Conclusion

In summary, the use of previously used NORG implants during the synchronization of ovulation protocol for TAI in beef heifers increases the occurrence of early ovulation and exhibits a greater efficiency when TAI is performed at 48 hours compared with 54 hours after implant removal. However, when using a new NORG implant, TAI can be performed either 48 or 56 hours after implant removal with no detrimental effect on the P/AI in beef heifers.

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